

CLAIMS

What is claimed is:

1. A method for the production of a carotenoid compound comprising:

- 5 (a) providing a transformed C1 metabolizing host cell comprising:
- (i) suitable levels of isopentenyl pyrophosphate; and
  - (ii) at least one isolated nucleic acid molecule encoding an enzyme in the carotenoid biosynthetic pathway
- 10 under the control of suitable regulatory sequences;
- (b) contacting the host cell of step (a) under suitable growth conditions with an effective amount of a C1 carbon substrate whereby an carotenoid compound is produced.

2. A method according to Claim 1 wherein the C1 carbon substrate

15 is selected from the group consisting of methane, methanol, formaldehyde, formic acid, methylated amines, methylated thiols, and carbon dioxide.

3. A method according to Claim 1 wherein the C1 metabolizing host cell is a methylotroph selected from the group consisting of

20 *Methylomonas*, *Methylobacter*, *Mehtylococcus*, *Methylosinus*, *Methylocyctis*, *Methylomicrobium*, *Methanomonas*, *Methylophilus*, *Methylobacillus*, *Methylobacterium*, *Hyphomicrobium*, *Xanthobacter*, *Bacillus*, *Paracoccus*, *Nocardia*, *Arthrobacter*, *Rhodopseudomonas*, *Pseudomonas*, *Candida*, *Hansenula*, *Pichia*, *Torulopsis*, and *Rhodotorula*

4. A method according to Claim 3 wherein C1 metabolizing host is

25 a methanotroph.

5. A method according to Claim 4 wherein the methanotroph is a methanotroph selected from the group consisting of *Methylomonas*, *Methylobacter*, *Mehtylococcus*, *Methylosinus*, *Methylocyctis*, *Methylomicrobium*, and *Methanomonas*.

30 6. A method according to Claim 2 wherein the C1 carbon substrate is selected from the group consisting of methane and methanol and the C1 metabolizing host cell is a methanotroph selected from the group consisting of *Methylomonas*, *Methylobacter*, *Mehtylococcus*, *Methylosinus*, *Methylocyctis*, *Methylomicrobium*, and *Methanomonas*.

35 7. A method according to Claim 6 wherein the methanotroph is a high growth methanotrophic strain which comprises a functional Embden-Meyerhof carbon pathway, said pathway comprising a gene encoding a pyrophosphate dependent phosphofructokinase enzyme.

8. A method according to Claim 7 wherein the gene encoding a pyrophosphate dependent phosphofructokinase enzyme is selected from the group consisting of:

- 5 (a) an isolated nucleic acid molecule encoding the amino acid sequence as set forth in SEQ ID NO:2;
- (b) an isolated nucleic acid molecule that hybridizes with (a) under the following hybridization conditions: 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS;
- 10 (c) an isolated nucleic acid molecule comprising a first nucleotide sequence encoding a polypeptide of at least 437 amino acids that has at least 63% identity based on the Smith-Waterman method of alignment when compared to a polypeptide having the sequence as set forth in SEQ ID NO:2; and
- 15 (d) an isolated nucleic acid molecule that is complementary to (a), (b) or (c).

9. A method according to Claim 7 wherein the high growth methanotrophic bacterial strain optionally contains at least one gene encoding a fructose biphosphate aldolase enzyme.

10. A method according to Claim 7 wherein the high growth methanotrophic bacterial strain optionally contains a functional Entner-Doudoroff carbon pathway.

11. A method according to Claim 8 wherein the high growth methanotrophic bacterial strain optionally contains at least one gene encoding a keto-deoxy phosphogluconate aldolase.

12. A method according to Claim 9 wherein the high growth methanotrophic bacterial strain is methylomonas 16a having the ATCC designation ATCC PTA 2402.

13. A method according to Claim 1 wherein the isolated nucleic acid molecule encodes a carotenoid biosynthetic enzyme selected from the group consisting of geranylgeranyl pyrophosphate (GGPP) synthase, phytoene synthase, phytoene desaturase, lycopene cyclase,  $\beta$ -carotene hydroxylase, zeaxanthin glucosyl transferase,  $\beta$ -carotene ketolase,  $\beta$ -carotene C-4 oxygenase,  $\beta$ -carotene desaturase, spheroidene monooxygenase, carotene hydratase, carotenoid 3,4-desaturase, 1-OH-carotenoid methylase, farnesyl diphosphate synthetase, and diapophytoene dehydrogenase.

14. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a geranylgeranyl pyrophosphate (GGPP) synthase selected from the group consisting of Genbank Acc #. AB000835, AB016043 AB019036,
- 5 AB027705, AB027706, AB016044, AB034249, AB034250, AF020041, AF049658, AF049659, AF139916, AF279807, AF279808, AJ010302, AJ133724, AJ276129, D85029, L25813, L37405, U15778, U44876, X92893, X95596, X98795, and Y15112
15. A method according to Claim 13 wherein the geranylgeranyl pyrophosphate (GGPP) synthase as the amino acid sequence as set forth in SEQ ID NO:26..
16. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a phytoene synthase selected from the group consisting of Genbank Acc #
- 15 AB001284, AB032797, AB034704, AB037975, AF009954, AF139916, AF152892, AF218415, AF220218, AJ010302, AJ133724, AJ278287, AJ304825, AJ308385, D58420, L23424, L25812, L37405, M38424, M87280, S71770, U32636, U62808, U87626, U91900, X52291, X60441, X63873, X68017, X69172, and X78814.
- 20 17. A method according to Claim 13 wherein the phytoene synthase as the amino acid sequence as set forth in SEQ ID NO:34.
18. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a phytoene desaturase selected from the group consisting of Genbank Acc #
- 25 # AB046992, AF039585, AF049356, AF139916, AF218415AF251014, AF364515, D58420, D83514, L16237, L37405, M64704, M88683, S71770, U37285, U46919, U62808, X55289, X59948, X62574, X68058, X71023, X78271, X78434, X78815, X86783, Y14807, Y15007, Y15112, Y15114, and Z11165
- 30 19. A method according to Claim 13 wherein the phytoene desaturase as the amino acid sequence as set forth in SEQ ID NO:32
20. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a lycopene cyclase selected from the group consisting of Genbank Acc #
- 35 AF139916, AF152246, AF218415, AF272737, AJ133724, AJ250827, AJ276965, D58420, D83513, L40176, M87280, U50738, U50739 U62808, X74599, X81787, X86221, X86452, X95596, and X98796.

21. A method according to Claim 13 wherein the lycopene cyclase as the amino acid sequence as set forth in SEQ ID NO:30

22. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a  $\beta$ -carotene hydroxylase selected from the group consisting of Genbank Acc # D58420, D58422, D90087, M87280, U62808, Y15112,

23. A method according to Claim 13 wherein  $\beta$ -carotene hydroxylase as the amino acid sequence as set forth in SEQ ID NO:36

24. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes zeaxanthin glucosyl transferase selected from the group consisting of Genbank Acc #. D90087, M87280, and M90698.

25. A method according to Claim 13 wherein zeaxanthin glucosyl transferase as the amino acid sequence as set forth in SEQ ID NO:28

26. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a  $\beta$ -carotene ketolase selected from the group consisting of Genbank Acc #. AF218415, D45881, D58420, D58422, X86782, and Y15112.

27. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a  $\beta$ -carotene ketolase having the amino acid sequence as set for the in SEQ ID NO:38.

28. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a  $\beta$ -carotene C-4 oxygenase selected from the group consisting of Genbank Acc #. X86782, and Y15112.

29. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a  $\beta$ -carotene desaturase selected from the group consisting of Genbank Acc #. AF047490, AF121947, AF139916, AF195507, AF272737, IFO13350, AF372617, AJ133724, AJ224683, D26095 U38550, X89897, Y15115, and PCC7210,

30. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a spheroidene monooxygenase selected from the group consisting of Genbank Acc #. AJ010302, Z11165, and X52291.

31. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a

carotene hydratase selected from the group consisting of Genbank Acc #. AB034704, AF195122, AJ010302, AF287480, U73944, X52291, Z11165, and Z21955.

32. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a carotenoid 3,4-desaturase selected from the group consisting of Genbank Acc #. AJ010302, X63204 U73944, X52291, and Z11165,

33. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a 1-OH-carotenoid methylase selected from the group consisting of Genbank Acc #. AB034704, AF288602, AJ010302, X52291 and Z11165.

34. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a farnesyl diphosphate synthetase selected from the group consisting of Genbank Acc #. AB003187, AB016094, AB021747, AB028044, AB028046, AB028047, AF112881, AF136602, AF384040, D00694, D13293, D85317, X75789, Y12072, Z49786, U80605, X76026, X82542 X82543, AF234168, L46349, L46350, L46367, M89945, NM\_002004, U36376, XM\_034497, XM\_034498, XM\_034499, and XM\_034500.

35. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a farnesyl diphosphate synthetase having the amino acid sequence as set forth in SEQ ID NO:20.

36. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a diapophytoene dehydrogenase enzyme as described by Genbank Acc #. X73889.

37. A method according to Claim 13 wherein the isolated nucleic acid molecule encoding a carotenoid biosynthetic enzyme encodes a diapophytoene dehydrogenase enzyme having the amino acid sequence selected from the group consisting of SEQ ID NO:22 and SEQ ID NO:24.

38. A method according to Claim 1 wherein said methanotrophic bacteria is *methylobionas* 16a ATCC PTA 2402.

39. A method according to Claim 1 wherein the suitable levels of isopentenyl pyrophosphate are provided by the expression heterologous upper pathway isoprenoid pathway genes.

40. A method according to Claim 39 wherein said upper pathway isoprenoid genes are selected from the group consisting of D-1-

deoxyxylulose-5-phosphate synthase (*Dxs*), D-1-deoxyxylulose-5-phosphate reductoisomerase (*Dxr*), 2C-methyl-d-erythritol cytidyltransferase (*IspD*), 4-diphosphocytidyl-2-C-methylerythritol kinase (*IspE*), 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (*IspF*), CTP synthase (*PyrG*), *lytB*, and *GcpE*.

41. A method according to Claim 40 wherein said gene encoding a D-1-deoxyxylulose-5-phosphate synthase (*Dxs*), encodes a polypeptide as set forth in SEQ ID NO:6

42. A method according to Claim 40 wherein said gene encoding a D-1-deoxyxylulose-5-phosphate reductoisomerase (*Dxr*), encodes a polypeptide as set forth in SEQ ID NO:8.

43. A method according to Claim 40 wherein said gene encoding a 2C-methyl-d-erythritol cytidyltransferase (*IspD*), encodes a polypeptide as set forth in SEQ ID NO:10.

44. A method according to Claim 40 wherein said gene encoding a 4-diphosphocytidyl-2-C-methylerythritol kinase (*IspE*), encodes a polypeptide as set forth in SEQ ID NO:12.

45. A method according to Claim 40 wherein said gene encoding a 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (*IspF*), encodes a polypeptide as set forth in SEQ ID NO:14.

46. A method according to Claim 40 wherein said gene encoding a CTP synthase (*PyrG*) encodes a polypeptide as set forth in SEQ ID NO:16.

47. A method according to Claim 40 wherein said gene encoding an activity for the production of dimethylallyl diphosphate (*lytB*) encodes a polypeptide as set forth in SEQ ID NO:18.

48. A method according to Claim 1 wherein the carotenoid compound is selected from the group consisting of Antheraxanthin, adonixanthin, Astaxanthin, Canthaxanthin, capsorubrin,  $\beta$ -cryptoxanthin, alpha-carotene, beta-carotene, epsilon-carotene, echinenone, gamma-carotene, zeta-carotene, alpha-cryptoxanthin, diatoxanthin, 7,8-didehydroastaxanthin, fucoxanthin, fucoxanthinol, isorenieratene, lactucaxanthin, lutein, lycopene, neoxanthin, neurosporene, hydroxyneurosporene, peridinin, phytoene, rhodopin, rhodopin glucoside, siphonaxanthin, spheroidene, spheroidenone, spirilloxanthin, uriolide, uriolide acetate, violaxanthin, zeaxanthin- $\beta$ -diglucoside, and zeaxanthin.

49. A method for the over-production of carotenoid production in a transformed C1 metabolizing host comprising:

- 5
- (a) providing a transformed C1 metabolizing host cell comprising:
- (i) suitable levels of isopentenyl pyrophosphate; and
- (ii) at least one isolated nucleic acid molecule encoding an enzyme in the carotenoid biosynthetic pathway under the control of suitable regulatory sequences; and
- (iii) either:
- 10 1) multiple copies of at least one gene encoding an enzyme selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (*Dxs*), D-1-deoxyxylulose-5-phosphate reductoisomerase (*Dxr*), 2C-methyl-d-erythritol cytidyltransferase (*IspD*), 4-diphosphocytidyl-2-C-methylerythritol kinase (*IspE*), 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (*IspF*), CTP synthase (*PyrG*) *lytB* and *gcpE*; or
- 15 2) at least one gene encoding an enzyme selected from the group consisting of D-1-deoxyxylulose-5-phosphate synthase (*Dxs*), D-1-deoxyxylulose-5-phosphate reductoisomerase (*Dxr*), 2C-methyl-d-erythritol cytidyltransferase (*IspD*), 4-diphosphocytidyl-2-C-methylerythritol kinase (*IspE*), 2C-methyl-d-erythritol 2,4-cyclodiphosphate synthase (*IspF*), CTP synthase (*PyrG*), *lytB* and *gcpE* operably
- 20 linked to a strong promoter.
- (b) contacting the host cell of step (a) under suitable growth conditions with an effective amount of a C1 carbon substrate whereby a carotenoid compound is over-
- 25 produced.
- 30 50. A method according to Claim 49 wherein the at least one gene encoding an enzyme of either part (a)(iii)(1) or (a)(iii)(2) encodes an enzyme selected from the group consisting of SEQ ID NO:6, 8, 10, 12, 14, 16, and 18.